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A glucagon-like peptide, structurally related to mammalian oxyntomodulin, from the pancreas of a holocephalan fish, *Hydrolagus colliei*

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The pancreatic islets of the holocephalan fishes contain, in addition to A-, B- and D-cells, X-cells, which are immunoreactive towards antisera directed against the *N*-terminal region of glucagon but not towards antisera directed against the *C*-terminal region. A 36-amino-acid-residue peptide was isolated from the pancreas of a holocephalan fish, the Pacific ratfish (*Hydrolagus colliei*), that shows homology (69%) to mammalian glucagon in its *N*-terminal region and is reactive towards an *N*-terminally directed antiserum. Reactivity towards *C*-terminally directed antisera is prevented by the presence of a 7-residue *C*-terminal extension to the glucagon sequence that shows limited homology to the *C*-terminal region of glucagon-37 (oxyntomodulin). It is proposed that this peptide represents a major storage product of the islet X-cell.

INTRODUCTION

The holocephalan fishes were the first class of vertebrate in evolution to develop a pancreatic gland with both exocrine and endocrine parenchyma (Fujita, 1962; Falkmer *et al.*, 1984). As in mammals, the endocrine tissue is localized to islets distributed throughout the pancreas, although isolated endocrine cells are found among the acinar tissue and in the epithelium of the pancreatic ducts. In the islets of the ratfish (*Hydrolagus colliei*), one such holocephalan fish, four types of endocrine cells have been described: A-cells containing glucagon, B-cells containing insulin, D-cells containing somatostatin, and X-cells (Patent, 1976; Falkmer, 1985). Cells containing pancreatic polypeptide were present only in the gut mucosa and occasionally in the pancreatic-duct epithelium. A further immunohistochemical study (Stefan *et al.*, 1981) has demonstrated that the X-cells are associated with an antigen that is detected with an antiserum that is directed against the *N*-terminal region of glucagon. The X-cells could not be immunostained with an antiserum directed against the *C*-terminal region of glucagon unless this immunodeterminant was first exposed by treatment of the sections with trypsin and carboxypeptidase B (Tager & Markese, 1979). This result led to speculation that the X-cell of the ratfish, like the L-cell in the intestinal mucosa of mammals, stores higher-M_r or precursor forms of glucagon (Stefan *et al.*, 1981). The isolation and characterization of insulin from the ratfish pancreas has been described (Conlon *et al.*, 1986). In the present study side fractions from that preparation were examined with regionally specific antisera to pig glucagon for the presence of peptides with glucagon-like immunoreactivity. A 36-amino-acid-residue peptide was identified, which, it is speculated, may represent a storage product of the X-cell.

EXPERIMENTAL

Ratfish (about 600 adult specimens; body wt. 1–3 kg) were collected at Bamfield Marine Station (Vancouver Island, B.C., Canada) during October and November. The extraction of pancreatic tissue (203 g) with ethanol/0.7 M-HCl (3:1, v/v) has been described previously (Conlon *et al.*, 1986). Ethanol was removed from the extract under reduced pressure, and the resulting solution was centrifuged (20000 *g* for 1 h at 4 °C). The supernatant from half of the extract was pumped at a flow rate of 1 ml/min on to eight Sep-pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and the effluent was freeze-dried.

Radioimmunoassay methods

Glucagon-like immunoreactivity was measured with an antiserum directed against a site in the *N*-terminal to central region of glucagon (probably residues 10–18) (Conlon *et al.*, 1985). This antiserum cross-reacts fully with highly purified glicentin (Thim & Moody, 1981) and with synthetic oxyntomodulin (glucagon-37) (Bataille *et al.*, 1982). These measurements were referred to as '*N*-terminal glucagon-like immunoreactivity' (N-GLI), and antisera of this specificity have been termed 'gut GLI cross-reactive'. Measurements were also made with the well-characterized Unger antiserum 30K (Faloona & Unger, 1974), directed against a site in the *C*-terminal region of glucagon (probably residues 24–29). The antiserum requires a free carboxy group on Thr-29 of glucagon for reactivity (Conlon, 1981). These measurements are termed '*C*-terminal glucagon-like immunoreactivity' (C-GLI), and antisera of this specificity have been described as 'pancreatic glucagon specific'. All data are expressed relative to a pig glucagon standard

Abbreviations used: N-GLI and C-GLI, *N*-terminal and *C*-terminal glucagon-like immunoreactivity respectively.

supplied by the Novo Research Institute, and [3-[^{125}I]iodotyrosine-10]glucagon (sp. radioactivity 74 TBq/mmol), supplied by Amersham Buchler (Braunschweig, Germany) was used as tracer. The minimum detectable concentrations of glucagon-like immunoreactivity were N-GLI, 22 pmol/l, and C-GLI, 11 pmol/l.

Purification of the peptide

The pancreatic extract, after concentration on Sep-pak cartridges, was chromatographed on a column (90 cm × 1.6 cm) of Sephadex G-50 (fine grade) equilibrated with 0.1 M-ammonium acetate, pH 6.8. Fractions (2.1 ml) were assayed at a dilution of 1:500 for glucagon-like immunoreactivity. Fractions with $K_{av.}$ between 0.23 and 0.42 (hatched bar in Fig. 1) were purified further by reverse-phase h.p.l.c. Samples, in 1 ml portions, were injected on to a Vydac 218 TP column (0.46 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.) eluted at 30 °C and 1.5 ml/min with a linear gradient (total volume 45 ml) formed from acetonitrile/water/trifluoroacetic acid (210:789:1, by vol.) and acetonitrile/water/trifluoroacetic acid (350:649:1, by vol.). U.v. absorbance was measured at 214 nm. The peak with glucagon-like immunoreactivity (Fig. 2) was freeze-dried and redissolved in 0.1% (v/v) trifluoroacetic acid (1 ml). The sample was injected in five 200 μl portions on to an Ultrapore RPSC column (0.46 cm × 7.5 cm) (Beckman/Altex, Berkeley, CA, U.S.A.) equilibrated with 0.1% trifluoroacetic acid at 30 °C and at 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 17.5% (v/v) over 5 min, followed by an increase to 28% (v/v) over 35 min. The u.v.-absorbing peak with glucagon-like immunoreactivity (Fig. 3) was purified to homogeneity on a Supelcosil LC-3DP column (0.46 cm × 25 cm) (Supelco, Bellefonte, PA, U.S.A.) equilibrated with 0.1% trifluoroacetic acid at 30 °C and at 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% (v/v) over 5 min, followed by an increase to 35% (v/v) over 35 min (Fig. 4).

Structural characterization of the peptide

The amino acid composition of the glucagon-related peptide was determined with a Durrum D-500 automatic analyser as previously described (Conlon & McCarthy, 1984). Approx. 2 nmol of peptide was used. The primary structure of the peptide was determined by automated Edman degradation in an Applied Biosystems model 470A gas-phase sequencer (Moody *et al.*, 1984). Approx. 5 nmol of peptide was used and the detection limit for amino acid phenylthiohydantoin derivatives was 0.5 pmol.

RESULTS

Glucagon-like immunoreactivities in ratfish pancreatic extracts

The ratfish pancreatic extract contained 690 pmol of glucagon-like immunoreactivity/g, measured with an antiserum directed against the *N*-terminal to central region of glucagon (N-GLI) and 67 pmol/g measured with an antiserum directed against the *C*-terminal region of glucagon (C-GLI). The immunoreactivity in serial dilutions of the pancreatic extracts gave rise to a line that was parallel to that for the pig glucagon standard in a radioimmunoassay with the *N*-terminally directed anti-

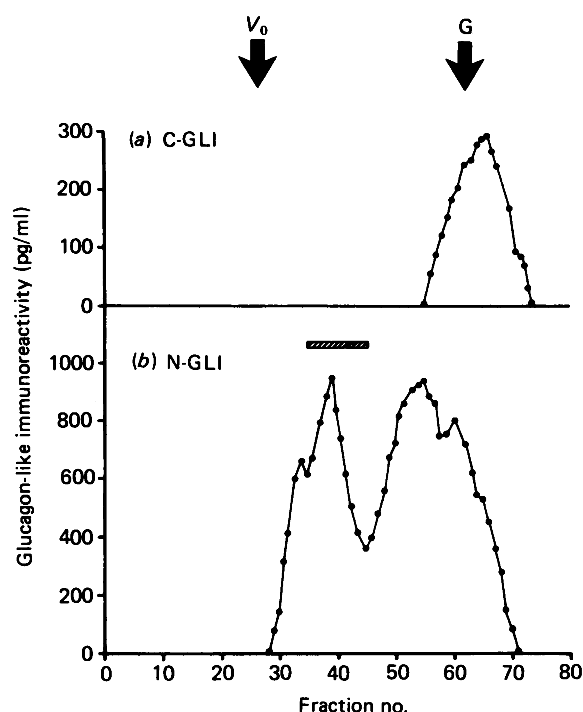

Fig. 1. Gel filtration (Sephadex G-50) of the glucagon-like immunoreactivity in an extract of ratfish pancreas measured with (*a*) an antiserum directed against the *C*-terminal region of glucagon and (*b*) an antiserum directed against the *N*-terminal region of glucagon

The arrows show the elution volume of pig glucagon (G) and the void volume of the column (V_0). The fractions indicated by the horizontal hatched bar were purified further.

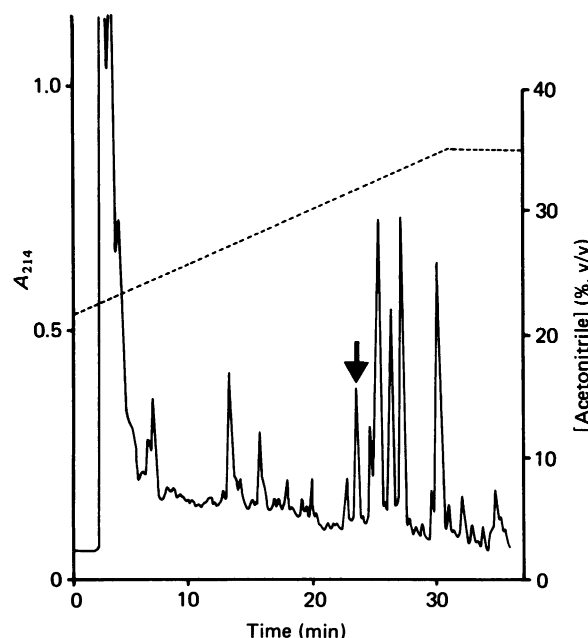

Fig. 2. Reverse-phase h.p.l.c. on a Vydac 218 TP column of a glucagon-related peptide from ratfish pancreas after partial purification by gel filtration (Fig. 1)

Details of the elution conditions are given in the text; -----, concn. of acetonitrile in the eluting solvent. The peak denoted by the arrow contained glucagon-like immunoreactivity and was purified further.

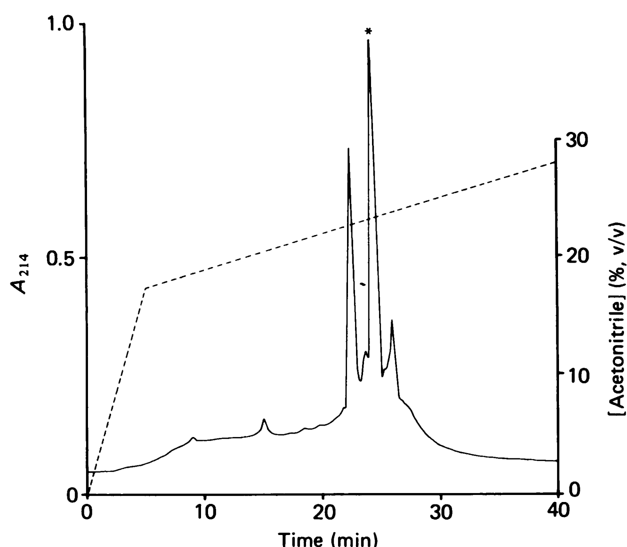

Fig. 3. Reverse-phase h.p.l.c. on an Ultrapore RPSC column of a glucagon-related peptide from ratfish pancreas

Elution conditions are given in the text; -----, concn. of acetonitrile in the eluting solvent. The peak denoted by the asterisk contained glucagon-like immunoreactivity and was purified further.

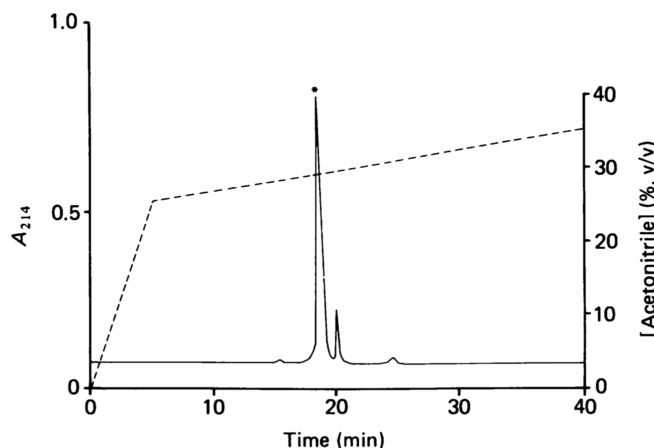

Fig. 4. Purification to homogeneity of a glucagon-related peptide from ratfish pancreas by reverse-phase h.p.l.c. on a Supelcosil LC-3DP column

The peptide is denoted by the asterisk and was characterized by amino acid composition and sequence analysis. -----, Concn. of acetonitrile in the eluting solvent.

serum. However, the immunoreactivity measured with the *C*-terminally directed antiserum did not dilute in parallel with the standard, so that the concentration of C-GLI measured by radioimmunoassay in the extract should be regarded only as an approximation. The N-GLI was resolved by gel filtration into multiple molecular forms, whereas the C-GLI was eluted from the column as a single peak with approximately the same elution volume as pig glucagon (Fig. 1). Only the major peak denoted by the hatched bar was selected for further purification.

H.p.l.c.

Fractions from gel filtration containing N-GLI (denoted by the hatched bar in Fig. 1) were chromatographed on a Vydac wide-pore C_{18} reverse-phase column (Fig. 2). The peak denoted by the arrow was associated with N-GLI and was purified further on an Ultrasphere C3 (n-propyldimethylsilylsilica) column. The peak was resolved into at least three components (Fig. 3), but only the major peak denoted by the asterisk was associated with N-GLI. The glucagon-related peptide was purified to homogeneity by chromatography on a Supelcosil LC-3DP (diphenylmethylsilylsilica) column (Fig. 4). The final yield of pure material, starting from 50% of the original extract, was approx. 12 nmol. This represents a yield of 17% of the N-GLI in the starting material.

Structural analysis

The amino acid composition of the glucagon-related peptide is shown in Table 1, and the results of the automatic Edman degradation are shown in Table 2. The average repetitive yield during operation of the sequencer was 90.3%. Unambiguous assignation of 36 amino acid reisdues was possible by Edman degradation.

Table 1. Amino acid composition of the glucagon-related peptide from the pancreas of the ratfish

The numbers in parentheses are the values from the sequence determination. Abbreviation: N.D., not determined.

Residue	Relative amount
Asx	6.76 (7)
Thr	3.91 (4)
Ser	4.00 (4)
Glx	1.37 (1)
Pro	0.04 (0)
Gly	2.10 (2)
Ala	1.17 (1)
Val	0.98 (1)
Met	0.00 (0)
Ile	1.04 (1)
Leu	3.37 (3)
Tyr	1.85 (2)
Phe	1.77 (2)
His	0.95 (1)
Lys	2.93 (3)
Arg	2.94 (3)
Trp	N.D. (1)

Agreement between the proposed sequence and the amino acid composition was good, indicating that the full sequence of the peptide has been obtained. An independent determination of the *C*-terminal residue was not carried out, and so the possibility that the sequence extends beyond residue 36 cannot be excluded entirely. The immunochemical properties of the peptide are consistent with the proposed structure. The strong homology with pig glucagon in the central region of the molecule (residues 8–15 and 17–18) accounts for the presence of N-GLI, and the presence of a *C*-terminal polypeptide extension to the Thr-29 residue accounts for the absence of C-GLI (Fig. 5).

Table 2. Automated Edman degradation of the glucagon-related peptide from the pancreas of the ratfish

Cycle no.	Amino acid phenylthiohydantoin	Yield (pmol)
1	His	856
2	Thr	1642
3	Asp	1742
4	Gly	3744
5	Ile	3412
6	Phe	3577
7	Ser	907
8	Ser	859
9	Asp	988
10	Tyr	1852
11	Ser	359
12	Lys	1862
13	Tyr	1270
14	Leu	1752
15	Asp	771
16	Asn	1720
17	Arg	672
18	Arg	744
19	Thr	460
20	Lys	1002
21	Asp	479
22	Phe	885
23	Val	798
24	Gln	822
25	Trp	266
26	Leu	670
27	Leu	757
28	Ser	175
29	Thr	177
30	Lys	294
31	Arg	211
32	Asn	502
33	Gly	283
34	Ala	372
35	Asn	400
36	Thr	81

DISCUSSION

The results of the present study confirm previous immunohistochemical observations (Stefan *et al.*, 1981) that the ratfish pancreas contains much higher concentrations of glucagon-like immunoreactivity measured with a *N*-terminally directed antiserum than with a *C*-terminally directed antiserum. One abundant peptide with *N*-terminal glucagon-like immunoreactivity was purified to homogeneity, and its structure is compared with pig glucagon, pig oxyntomodulin (glucagon-37) and glucagon from an elasmobranchian fish, *Torpedo marmorata* (Conlon & Thim, 1985), in Fig. 5. The *N*-terminal region of the ratfish peptide (residues 1–29) shows homology to glucagon from the pig (69%) and *Torpedo* (76%), and the *C*-terminal extension to the glucagon sequence shows identity in four out of seven amino acid residues with the corresponding extension in pig oxyntomodulin. The holocephalan fishes are phylogenetically related to the elasmobranchian fishes, but diverged from the line of evolution leading to contemporary sharks and rays at least 250 million years ago. Nevertheless, the unusual structural features of Asn-16 and Lys-20 found in *Torpedo* glucagon are also found in the ratfish peptide. Glucagon-37 was first isolated as a contaminant in a commercially available mixture of pig and bovine glucagons (Tager & Steiner, 1973), and was subsequently purified from extracts of pig small intestine (Bataille *et al.*, 1982). The concentration of this component in the pancreas of most mammalian species studied is low (Conlon, 1981), but in the guinea pig a difference in the primary structure of glucagon (Conlon *et al.*, 1985; Huang *et al.*, 1986) is accompanied by a much higher concentration of glucagon-37 (Conlon *et al.*, 1985).

The immunohistochemical study by Stefan *et al.* (1981) demonstrated that the ratfish pancreatic islets contained two populations of cells with different glucagon immunoreactivity. It is proposed that the 36-amino-acid-residue peptide isolated in this study is a storage product of the X-cells, i.e. those cells that may be stained with the *N*-terminally directed serum but not the *C*-terminally directed antiserum to glucagon. The gel-filtration profile shown in Fig. 1 indicates that the X-cell may also store, in lower concentration, higher-M_r glucagon-like peptides that may be more similar in structure to pig glicentin. Attempts to purify the glucagon-like peptide with *C*-terminal immunoreactivity (Fig. 1) have not yet been successful. However, since the *C*-terminally directed antiserum will only recognize glucagon-like peptides with a free Thr-29 residue (Conlon, 1981), it is probable that this peptide represents residues 1–29 of the larger peptide purified in the present study. The presence of amino acid substitutions at positions 27 (leucine for methionine) and 28 (serine for asparagine) in the ratfish sequence probably means that the concentration of *C*-terminal immunoreactivity in the ratfish pancreas, measured by radioimmunoassay and shown in Fig. 1, is greatly underestimated.

Mutations in the guinea-pig preproglucagon gene are

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                                  5         10        15        20        25        30        35
Ratfish peptide          H T D G I F S S D Y S K Y L D N R R T K D F V Q W L L S T K R N G A N T

Pig glucagon             - S Q - T - T - - - - - - - - S - - A Q - - - - - - M N -

Pig oxyntomodulin        - S Q - T - T - - - - - - - - S - - A Q - - - - - - M N - - - - K N - I A

T. marmorata glucagon    - S E - T - T - - - - - - - - - - - A - - - - - - - M N -
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Fig. 5. Comparison of the primary structures of the glucagon-related peptide from the ratfish, pig glucagon and oxyntomodulin, and glucagon from the elasmobranchian fish *T. marmorata*

largely restricted to the *C*-terminal region of the glucagon sequence (Seino *et al.*, 1986). As this region is involved in binding to the receptor, it has been speculated that the changes in structure are an adaptive response to divergence in primary structure of guinea-pig insulin. The amino acid sequence of ratfish insulin shows some unique structure features, in particular an extension to the B-chain, which are expected to result in marked changes in biological potency (Conlon *et al.*, 1986). It is therefore tempting to speculate that, in the ratfish also, changes in the pathway of post-translational processing of proglucagon and substitutions in the *C*-terminal region of glucagon are an adaptive response to overcome the metabolic imbalance that would result from an insulin and a glucagon of appreciably different potencies in the same species.

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